**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C07H 21/00 | A2

(11) International Publication Number: **WO 00/53616**

(43) International Publication Date: 14 September 2000 (14.09.00)

(21) International Application Number: PCT/GB00/00843

(22) International Filing Date: 7 March 2000 (07.03.00)

(30) Priority Data:
99301764.9 9 March 1999 (09.03.99) EP

(71) Applicant *(for all designated States except US)*: AMERSHAM PHARMACIA BIOTECH UK LTD [GB/GB]; Amersham Laboratories, White Lion Road, Amersham, Buckinghamshire HP7 9LL (GB).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: ROSLER, Angelika, Josefine [DE/GB]; The White House, Little Missenden, Amersham, Buckinghamshire HP7 0QX (GB).

(74) Agent: HAMMER, Catriona, MacLeod; Amersham Pharmacia Biotech UK Ltd, Amersham Laboratories, White Lion Road, Amersham, Buckinghamshire HP7 9LL (GB).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published**

*Without international search report and to be republished upon receipt of that report.*

(54) Title: IMMOBILISING POLYNUCLEOTIDES

(57) Abstract

A method of immobilising a nucleotide or polynucleotide on a solid support, comprises: providing the solid support having a functionalised surface of the formula B – S – S – L where B is the solid support and L is a leaving group; providing a nucleotide or polynucleotide comprising at least one phosphorothioate group; and coupling the two together by means of a sulphide exchange reaction. The product has the structure B – S – S – $P(O)R^1R^2$, where – $POR^1R^2$ represents a nucleotide or a polynucleotide. Arrays of the immobilised polynucleotides are useful for performing APEX or SBH assays.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **AL** | Albania | **ES** | Spain | **LS** | Lesotho | **SI** | Slovenia |
| **AM** | Armenia | **FI** | Finland | **LT** | Lithuania | **SK** | Slovakia |
| **AT** | Austria | **FR** | France | **LU** | Luxembourg | **SN** | Senegal |
| **AU** | Australia | **GA** | Gabon | **LV** | Latvia | **SZ** | Swaziland |
| **AZ** | Azerbaijan | **GB** | United Kingdom | **MC** | Monaco | **TD** | Chad |
| **BA** | Bosnia and Herzegovina | **GE** | Georgia | **MD** | Republic of Moldova | **TG** | Togo |
| **BB** | Barbados | **GH** | Ghana | **MG** | Madagascar | **TJ** | Tajikistan |
| **BE** | Belgium | **GN** | Guinea | **MK** | The former Yugoslav Republic of Macedonia | **TM** | Turkmenistan |
| **BF** | Burkina Faso | **GR** | Greece | | | **TR** | Turkey |
| **BG** | Bulgaria | **HU** | Hungary | **ML** | Mali | **TT** | Trinidad and Tobago |
| **BJ** | Benin | **IE** | Ireland | **MN** | Mongolia | **UA** | Ukraine |
| **BR** | Brazil | **IL** | Israel | **MR** | Mauritania | **UG** | Uganda |
| **BY** | Belarus | **IS** | Iceland | **MW** | Malawi | **US** | United States of America |
| **CA** | Canada | **IT** | Italy | **MX** | Mexico | **UZ** | Uzbekistan |
| **CF** | Central African Republic | **JP** | Japan | **NE** | Niger | **VN** | Viet Nam |
| **CG** | Congo | **KE** | Kenya | **NL** | Netherlands | **YU** | Yugoslavia |
| **CH** | Switzerland | **KG** | Kyrgyzstan | **NO** | Norway | **ZW** | Zimbabwe |
| **CI** | Côte d'Ivoire | **KP** | Democratic People's Republic of Korea | **NZ** | New Zealand | | |
| **CM** | Cameroon | | | **PL** | Poland | | |
| **CN** | China | **KR** | Republic of Korea | **PT** | Portugal | | |
| **CU** | Cuba | **KZ** | Kazakstan | **RO** | Romania | | |
| **CZ** | Czech Republic | **LC** | Saint Lucia | **RU** | Russian Federation | | |
| **DE** | Germany | **LI** | Liechtenstein | **SD** | Sudan | | |
| **DK** | Denmark | **LK** | Sri Lanka | **SE** | Sweden | | |
| **EE** | Estonia | **LR** | Liberia | **SG** | Singapore | | |

# IMMOBILISING POLYNUCLEOTIDES

This invention is concerned with the immobilisation of nucleotides and polynucleotides on solid surfaces. Immobilised polynucleotides are used in immunoassays and ELISA assays and also in hybridisation assays. More particularly, arrays of immobilised oligonucleotides are being widely used to compare target nucleic acids and indeed for de novo sequencing. For these purposes, it is essential that the polynucleotides be immobilised in forms capable of undergoing hybridisation or other reactions, and by links that are not labile under conditions likely to be encountered in hybridisation or other assays.

Disulphide bonds are very widely used to covalently bind proteins and other biomolecules on solid surfaces. However, thiol derivatives can easily form symmetric dimers in the presence of air oxygen. M Chassignol and N T Thuong have reported (Tetrahedron Letters, 39, 1998, 8271-4) the synthesis of conjugated oligonucleotides via a phosphodisulphide bridge, obtained by reacting in solution a terminal phosphorothioate oligonucleotide with a alkyl-2-pyridyldisulphide derivative. Using this strategy, formation of symmetrical oligonucleotide disulphide by oxidation was avoided.

In one aspect the invention provides a method of immobilising a nucleotide or polynucleotide on a solid support, by providing a nucleotide or a polynucleotide comprising at least one phosphorothioate group and coupling this to the support as defined by means of a sulphide exchange reaction.

In another aspect the invention provides a product having the structure:

$$B - S - S - P(O)R^1R^2$$

where B is the solid support and $R^1$ and $R^2$ are such that $-POR^1R^2$ represents a nucleotide or a polynucleotide.

In yet another aspect the invention provides an array of polynucleotides immobilised at spaced locations on a surface of a support as defined.

The support may be massive, e.g. a surface of a reaction vessel or the wells of a microtitre plate, or may be particulate. Of particular interest for arrays are flat surfaces which may be porous or non-porous. The material of the support should be stable against oxidation and hydrolysis, and may be inorganic e.g. silicon or titanium dioxide or aluminium oxide or preferably glass; or organic e.g. polystyrene, cellulose, polyamide and others.

The support preferably has a functionalised surface comprising groups of formula – S – S – L, where L is a leaving group, i.e. a group which is readily replaced by a nucleotide or a polynucleotide. Examples of suitable leaving groups are 2-pyridyl and 2-(5-nitropyridyl). Other suitable groups are well known in the field, see Bioconjugate Techniques by Greg T Hermanson, Academic Press, 1996.

A solid support having a functionalised surface of this kind can be prepared in various ways. For example, a solid support having surface hydroxyl groups can be reacted with a mercaptosilane such as (3–mercaptopropyl)–trimethoxysilane. This results in a surface carrying thiol groups, which is reacted with a di-(organic)disulphide, where one or both of the organic groups is a leaving group, to give the required functionalised surface. In another example, a surface carrying amino groups such as aminosilanased glass or amylated polypropylene may be reacted with for example 3,3'-dithiopropionic acid in the presence of a coupling reagent such as 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC) or O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), followed by reduction with dithiothreitol and reaction with a di-(organic)disulphide to yield the required functionalised

surface. A disulphide functionalised surface may also be prepared by reacting a surface carrying amino groups with 2-carboxyethyl-2'-(leaving group)disulphide in the presence of a coupling reagent such as EDC or HBTU.

The disulphide functionality – S – S – L may be linked to the solid support through a linker, for example an organic chain of up to 60 atoms selected from C, O, N and P and/or one or more silicon or silicate moieties. The nature and existence of such linkers is well known in the art and is not material to the present invention. The solid support B is herein deemed to include an optional linker through which the disulphide functionality – S – S – L is immobilised.

The solid support having a functionalised surface is designed to immobilise a nucleotide, which term is used to include nucleotide analogues, or a polynucleotide, which term is used to include oligonucleotides of natural or synthetic origin and which may contain nucleotide analogue residues; and polynucleotides which may be single-stranded or double-stranded and may be RNA or cDNA or DNA of genomic or other origin or PCR fragments and may include nucleotide analogue residues. A nucleotide analogue is a nucleotide modified in the base and/or sugar and/or phosphate moiety and is capable of forming base-pair hybrids. Although the length of the polynucleotide is immaterial, the invention is likely to be of particular interest for the immobilisation of oligonucleotides and of PCR fragments.

The nucleotide or polynucleotide to be immobilised is modified by being provided with a phosphorothioate group (also known as a thiophosphate group). This may preferably be done by replacing a 5'-terminal or 3'-terminal phosphate group – $PO_4H$ with a phosphorothioate group – $PO_3SH$. The modified nucleotide or polynucleotide is contacted with the functionalised surface of the solid support under conditions to couple the two together by means of a sulphide exchange reaction. Preferred reaction conditions are as follows. The temperature should be

from 0 to 80°C, preferably from ambient to 50°C. The modified nucleotide or polynucleotide is provided in aqueous solution preferably containing an organic water-miscible polar liquid such as ethylene glycol or dimethylsulphoxide (DMSO) which reduces the rate of evaporation. The proportion of such organic liquid may be up to 50% or even higher. The pH is preferably in the range 1.5 to 8, particularly 3 to 6; above pH 8 covalent binding by means of disulphide links begins to be swamped by non-specific binding. The nucleotide or polynucleotide solution may be maintained in contact with the functionalised surface of the solid support for long enough to effect coupling, e.g. 1 minute to 24 hours, if necessary using a high humidity atmosphere to delay evaporation. Then the support is washed to remove unreacted nucleotides and polynucleotides and is ready for use.

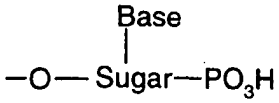
pH control may be effected by means of buffers commonly used with nucleic acids, for example sodium citrate/citric acid buffer and imidazole buffer. The nucleotide or polynucleotide concentration is preferably 0.1 to 100 μM.

In order to form arrays, e.g. for APEX (arrayed primer extension WO 95/00699) or SBH (sequencing by hybridisation) assays, different polynucleotides may be spotted on to the functionalised surface of the solid support at spaced locations using a spotter of the kind that is commercially available.

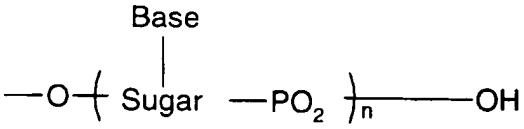
There results a product having the structure

$$B - S - S - P(O)R^1R^2,$$

where B is a solid support including an optional linker. The group $-P(O)R^1R^2$ represents a nucleotide or polynucleotide moiety. Thus in the case of a nucleotide, $R^1$ represents an oxygen atom or a hydroxyl group and $R^2$ represents a moiety



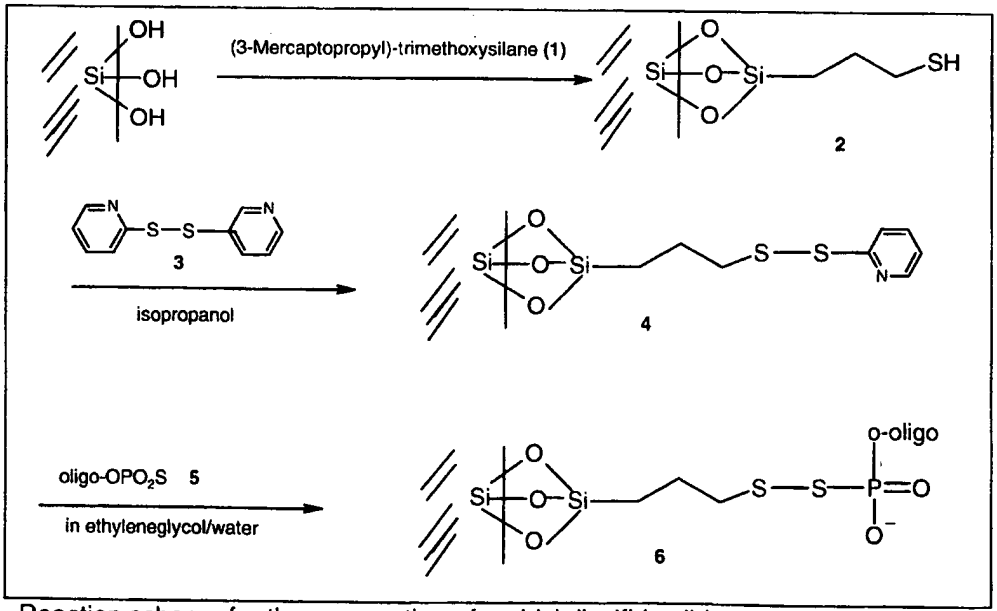
In the case of a polynucleotide which carries a 5'-terminal or a 3'-terminal phosphorothioate group, $R^1$ is oxygen or hydroxyl and $R^2$ is a moiety




where n is an integer of at least 2.

A polynucleotide may be immobilised on a solid support by a single disulphide bond or by a plurality of such bonds which may be at the end or intermediate the ends of the polynucleotide chain. The bonds are stable to the conditions commonly encountered in hybridisation or other assays, but are labile under reducing conditions.

The following examples illustrate the invention. Example 1 describes a typical procedure. Examples 2 and 3 describe specific comparative experiments whose results are reported. The chemical reactions involved are illustrated in the following reaction scheme.




Reaction scheme for the preparation of pyridyl disulfide slides and the binding of phosphorothioates to pyridyl disulfide slides

Reference is directed to Figure 1 which consists of three bar charts showing signal intensities of the Cy3 hybridisation signal for 3 repeat slides for 5' phosphorothioate and 5' phosphate modified oligonucleotides spotted at different pH values

## EXAMPLE 1

Typical procedure used for the method of this invention: Slides preparation: 25 precleaned glass microscope slides are put into a metal rack. A 5% (v/v) solution of (3-Mercaptopropyl)trimethoxysilane) in dry toluene is prepared and the slides soaked in this solution for 6 hours. The slides are rinsed 3 times with dry toluene followed by isopropanol. The slides are soaked in a solution of 6.66 g/l 2,2'-dipyridyl disulfide in isopropanol. The slides are washed 3 times with isopropanol and were allowed to dry.

For the immobilisation oligonucleotide probes are dissolved in 0.26 M citrate buffer pH 4 and mixed 1:1 with ethylene glycol or DMSO (final concentration 1 to 10 µM). The oligonucleotide probes are spotted on the disulfide modified surface (typically 0.8 nl per spot) using a micro array spotter. The spotted surface is kept at a humidity of 45% at room temperature for 4 hours, washed with distilled water and allowed to dry.

Hybridisation is carried out with Cy3 end labelled complementary oligonucleotide at a concentration of 0.3 µM. 30 µl Target solution (oligonucleotide No 16, 0.3 µM in hybridisation buffer) is applied to the slide surfaces and covered with a coverslip. Preferred hybridisation buffer is 7% (w/v) Sodium N-lauroyl sarosinate in 5x SSC buffer. The slides are kept in a humid atmosphere in a sealed box at 4°C overnight. The slides are washed twice for 5 minutes with ice-cold 10 x SSC buffer. Hybridised oligonucleotide is visualised by scanning with a micro array scanner for the fluorescence signal of the target.

Oligonucleotides: All oligonucleotides are purchased commercially.

**Oligonucleotides**

No 1: 5' - $PO_4$-GTA TGA AG

No 2: 5' - $NH_2$-GTA TGA AG

No 3: 5' - HS-GTA TGA AG

No 158: 5' - $PO_3S$-GTA TGA AG

No 16: 5' Cy3-CTT CAT AC

Oligonucleotide solutions of 2μM and 10 μM were prepared in water.

## EXAMPLE 2

Disulfide functionalised glass plates were prepared as described in Example 1.

Oligonucleotides, with a 5'-amino, 5'-phosphate, 5'-thiol or 5'-phosphorothioate modification were dissolved in 0.8 M imidazole pH 6 (25%) or 0.8M carbonate buffer pH 9 and ethyleneglycol (25%) and water (50%) to a final concentration of 0.5 μM.

The oligonucleotides were spotted with a microarray spotter on the prepared glass slides. The slides were incubated at 50°C in a humidity chamber. After 4h the slides were washed wit 15% ammonia, rinsed thoroughly with water and dried.

Following hybridisation with a complementary Cy3 labelled oligonucleotide, the Cy3 fluorescence signal was measured with a micro array scanner.

No specificity was found when the oligonucleotides were spotted out of carbonate buffer pH 9. At this pH all of the modified oligonucleotides showed the same signal intensity.

A high Cy3 fluorescence signal could be seen for 5' phosphorothioate oligonucleotide after hybridisation with Cy3 labelled complementary oligonucleotide when spotted out of imidazole buffer at pH 6.

However, no signal could be seen for the 5'- phosphate,

5'-amino or 5'-thiol oligonucleotide when spotted out of imidazole buffer at pH of 6.

This indicates specific binding of the 5' phosphorothioate oligonucleotide at pH 6 in imidazole buffer.

## EXAMPLE 3

Disulfide functionalised glass plates were prepared as described in Example 1.

Octameric oligonucleotides, with a 5'-phosphate or 5'-phosphorothioate modification (10μM), were dissolved to a final concentration of 2.5 μM in citrate buffers at different pH 1.5 to pH 7.8 (25%), ethyleneglycol or DMSO (25%) and water (25%). The oligonucleotides were spotted with a microarray spotter on the prepared glass slides. The slides were kept for 2 hours at room temperature at a humidity of 45%. The slides were rinsed with water and allowed to dry. Following hybridisation with a complementary Cy3 labelled oligonucleotide, the Cy3 fluorescence signal was measured with a micro array scanner.

Figure 1 shows signal intensities of the Cy3 hybridisation signal for 3 repeat slides.

An increase in Cy3 hybridisation signal intensity was found for the 5'-phosphorothioate oligonucleotide with decreasing pH from pH 7.8 to pH 4.

Non-specific binding of 5'-phosphate oligonucleotide decreased with decreasing pH as can be seen in Figure 1.

## Additional Oligonucleotides

217: 5'-ATT-CGC-GGT-ATT-CTG-GTA-TGA-AGC-TTT-TGG-ATC-CTG-TTA-ATG-AGT-TAG-TA-3'

219: 5'-$NH_2$-TAA-CTC-ATT-AAC-AGG-ATC-3'

218: 5'-$PO_3S$-TAA-CTC-ATT-AAC-AGG-ATC-3',

Oligonucleotide solutions of 100μM were prepared. Sequence 218 was chosen to bind specifically to disulfide functionalised solid support and to give strand extension when hybridised to Sequence 217 and incubated with Cy3 labelled ddCTP and Thermosequenase.

## EXAMPLE 4

Disulfide functionalised glass plates were prepared as described in Example 1.

Oligonucleotides 218 and 219 were dissolved in 0.8M Citrate buffer pH4 (25%), DMSO (50%) and water (25%) to a final concentration of 2.5 μM. The oligonucleotides were spotted with a micro array spotter on the prepared glass slides. The slides were incubated at 25°C and at a humidity of 40% for 4 hours. The slides were rinsed with water, treated for 15 minutes with 17% ammonia, washed with water (4X) followed by isopropanol and were dried.

Thermosequenase: Amersham Pharmacia Biotech, DDT free in 20 mM Tris/HCl pH8.5, 0.1 mM EDTA, 100 mM KCl, 0.5% Tween 20, 0.5% NP-40, 50% Glycerol. Buffer exchange was achieved by dialysis using Spectra/POR Steril Dispo Dialysers (Sigma 135488); 10X Thermosequenase reaction buffer: 260mM Tris/HCl pH9, 65 mM $MgCl_2$; Thermosequenase dilution buffer: 10 mM Tris/HCl pH8, 0.5% Tween 20, 0.5% NP-40.

For arrayed primer extension (APEX) reaction 1μl oligonucleotide 217 (100μM), 3μl Thermosequenase reaction buffer and 33μl water were combined per sample. The samples were heated to 100ºC for 5 minutes and put on ice. 1μl Cy3-ddCTP (100μM) and 2μl Thermosequenase (diluted to 3.3 U/μl) were added to the samples, the samples mixed quickly and 35 μl of each added to each array. The array was covered with a cover-slip and incubated for 40 minutes at 48ºC. The glass slides were washed twice with 2X SSC buffer followed by a rinse with water and isopropanol and were allowed to dry. The Cy3-fluorescence

signal was visualised by scanning with a Molecular Dynamics micro array scanner for the fluorescence signal of extended primer.

The Cy3-fluorescence signal after primer extension (APEX) of 5' phosphorothioate modified oligonucleotides, immobilised to pyridyl-disulfide slides, was found to be highly specific when the oligonucleotide was immobilised to the surface at a pH of 4. No signal was found for the 5'-amino oligonucleotide.

**Result Example 4**

Oligonucleotides with or without a terminal phosphorothioate function were applied to a pyridyl disulfide functionalised surface following procedures described in example 1. Oligonucleotide 218 carries a terminal phosphorothioate function and therefore binds covalently to the disulfide functionalised solid surface. Oligonucleotide 219 is not modified with a phosphorothioate group and no attachment to the solid surface was expected for this oligonucleotide (219) at pH4. After hybridisation of the glass slides with oligonucleotide 217 arrayed primer extension (APEX) was carried out on the solid surface. In the presence of oligonucleotide 217 both sequences 218 and 219 are capable of being extended with Thermosequenase / Cy3-ddCTP if present. As 219 is not attached to the surface no target was available for hybridisation and hence no signal seen under APEX conditions. 'APEX' signals were found for Sequence 218 only. This proves availability of disulfide functionalised solid supports to covalent attachment of target oligonucleotide, hybridisation and primer extension in the presence of Thermosequenase and Cy3 labelled ddCTP.

**CONCLUSIONS**

It was concluded from the above that the attachment of phosphorylated oligonucleotides to disulfide functionalised solid surfaces is highly specific. Using 5' phosphorothioate oligonucleotides it was found that optimal results could be achieved when the oligonucleotide was

applied to the surface at pH4 to pH6 in 0.2M citrate buffer or 0.2M imidazole/HCl mixed with ethyleneglycol or DMSO at a concentration of 2.5µM.

The immobilised oligonucleotides retained their ability for hybridisation and recognition by polymerases such as Thermosequenase.

This method presents a simple and efficient method to covalently attach nucleic acids to solid surfaces via formation of a phosphodisulfide bridge.

## CLAIMS

1. A product having the structure:

$$B - S - S - P(O)R^1R^2$$

where B is the solid support, and $R^1$ and $R^2$ are such that $-POR^1R^2$ represents a nucleotide or a polynucleotide.

2. The product of claim 1, wherein B is of glass, cellulose, polyamide, titanium dioxide or aluminium oxide.

3. An array of polynucleotides immobilised at spaced locations on a surface of a support, wherein each polynucleotide is covalently linked to the support by means of a link $B - S - S - P(O)R^1R^2$, where B is the solid support, and $R^1$ and $R^2$ are such that $-POR^1R^2$ represents the polynucleotide.

4. A method of immobilising a nucleotide or polynucleotide on a solid support, which method comprises: providing the solid support having a functionalised surface of the formula $B - S - S - L$ where B is the solid support and L is a leaving group; providing a nucleotide or polynucleotide comprising at least one phosphorothioate group; and coupling the two together by means of a sulphide exchange reaction.

5. The method of claim 4, wherein a polynucleotide has a 3'- or 5'- terminal phosphorothioate group.

6. The method of claim 4 or claim 5, wherein the nucleotide or polynucleotide is provided in solution at a pH of 1.5 – 8.

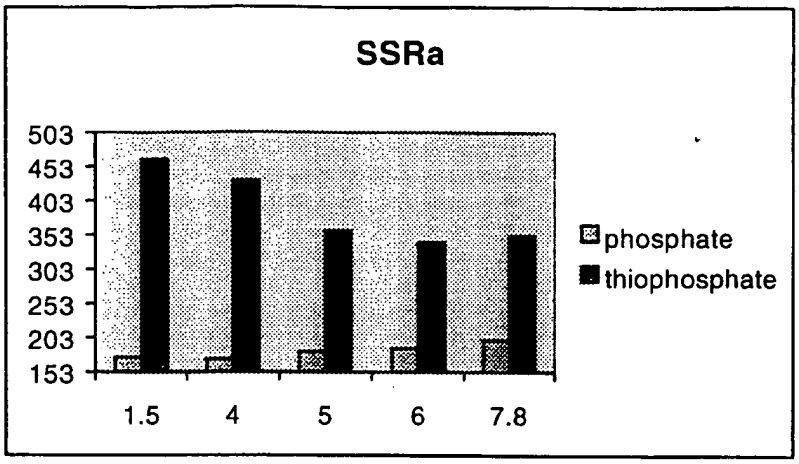
7. The method of any one of claims 4 to 6, wherein the nucleotide or polynucleotide is provided in an aqueous medium comprising a water-miscible liquid less volatile than water.

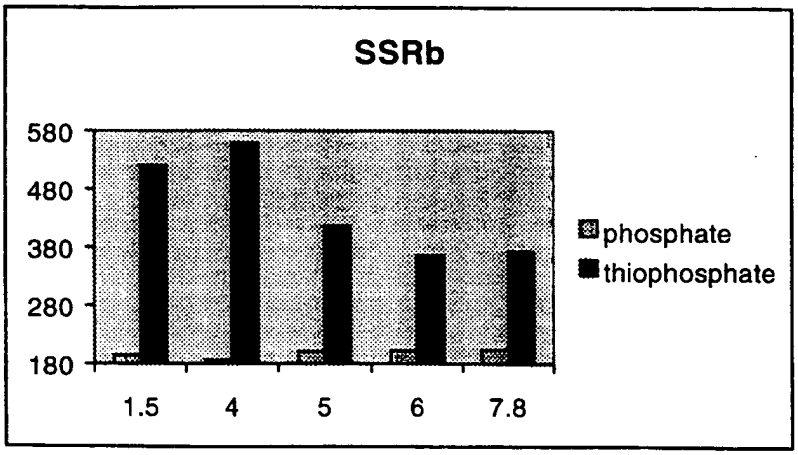
8. The method of any one of claims 4 to 7, wherein different nucleotides or polynucleotides are coupled at spaced locations on the surface of the support in the form of an array.

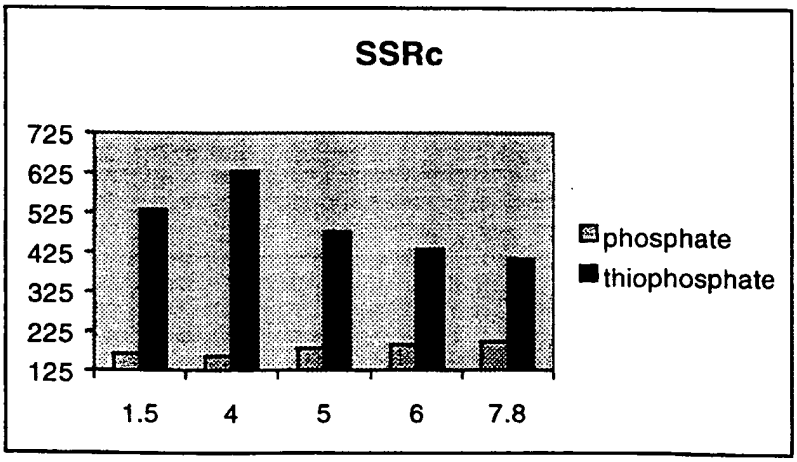
9. The method of claim 8, wherein nucleotides or polynucleotides are applied at spaced locations on the surface of the support by means of a microarray spotter.

10. The method of any one of claims 4 to 9, wherein L is pyridyl or 5-nitropyridyl.

11. A method of performing an arrayed primer extension assay or a sequencing by hybridisation assay, which method comprises applying a polynucleotide in solution to an array of polynucleotides, wherein the array of polynucleotides is as defined in claim 3.


SSRa
503
453
403
353
303
253
203
153
1.5
4
5
6
7.8
phosphate
thiophosphate


SSRb
580
480
380
280
180
1.5
4
5
6
7.8
phosphate
thiophosphate


SSRc
725
625
525
425
325
225
125
1.5
4
5
6
7.8
phosphate
thiophosphate